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Over expression of Wnt proteins has long been know to lead to mammary hyperplasia and neoplasia in the mouse mammary gland. Unregulated Wnt signaling may also contribute to the formation of human breast tumours as over expression of Wnt proteins and nuclear localization of β-catenin has been reported. The experiments in this proposal are designed to identify the receptor proteins responsible for transduction of oncogenic Wnt signals in mammary cells. Possible candidates are members of the Frizzled, Notch and LRP families of transmembrane proteins as all have been suggested to act as Wnt receptors.

A survey of the Frizzled, Notch and LRP proteins expressed in the mature virgin mammary gland of the mouse has been conducted to identify candidate receptors. Currently the ability of these proteins to interact physically with Wnt ligands is being tested in cross-linking and co-immunoprecipitation experiments. Also the ability of these proteins to transduce, and of dominant negative forms to attenuate, a Wnt signal is being evaluated in a functional assay for Wnt signaling. Finally, a mammary specific transgene encoding a dominant negative Wnt receptor has been produced. This will be introduced into mice by pronuclear injection and tested for its ability to antagonize extracellular Wnt signals in mice and to prevent the onset of mammary hyperplasia and tumorigenesis caused by the Wnt-1 oncogene.

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Introduction

Proteins of the Wnt family of secreted intercellular signaling factors regulate the proliferation and differentiation of a variety of different tissues, including the mammary gland (Cadigan and Nusse, 1997). In addition they have been shown to lead to hyperplasia and subsequently neoplasia when over-expressed in the mouse mammary gland (Nusse and Varmus, 1992). Over-expression of Wnt proteins has been reported in human breast tumors and unregulated Wnt signaling has been observed in other human cancers, such as colorectal tumors, implying that Wnt signaling plays a crucial role human cancer (Polakis, 2000). While much progress has been made in elucidating intracellular components of the Wnt signaling pathway, the precise nature of the receptors for mitogenic Wnt signals in the mammary gland has remained unclear. Characterization of these receptors is crucial for understanding how Wnt signals cause hyperplasia and tumorigenesis in mammary tissues and for the design of drugs and treatment strategies to antagonize Wnt signals for the prevention and treatment of breast cancer. There is considerable evidence that members of the Frizzled family of seven transmembrane domain proteins are receptors for Wnt ligands, but proteins of the Notch and LRP families have also been proposed to act as Wnt receptors (Martinez Arias et al., 1999; Bejsovec, 2000). All three gene families contain multiple members in mammalian species and their functional relationships with the many different mammalian Wnt proteins remain unclear. The experiments in this proposal will identify the receptors responsible for the transduction of oncogenic Wnt signals in mammary cells and will exploit that knowledge in a strategy to antagonize Wnt signals in vivo.

Body

Progress during the first year of the grant will be described in this report and will be summarized for each specific aim in the original proposal. Due to technical problems with experiments described in the second aim of the proposal, we have initiated experiments described in the third and fourth aims to ensure they are completed during the tenure of the grant.

Since the submission of the original proposal a member of the LRP (low density lipoprotein receptor related protein) protein family, LRP6, has been shown to be essential for Wnt signalling (Pinson et al., 2000). As both LRP6 and its highly related orthologue, LRP5, are

transmembrane proteins, it has been suggested that they may be components of the Wnt receptor. Therefore we have included these proteins in our analysis.

I. Expression analysis of *frizzled*, *Notch* and *LRP* genes in the mammary gland.

The aim of this section was to determine which of the *frizzled*, *Notch* and *LRP* genes are expressed in the mammary gland of mature virgin mice and hence which of these family members are candidates for transducing oncogenic Wnt signals. This has been completed and the results are described below (see table 1). However as the virgin mammary gland consists mainly of stromal tissue, this analysis may not identify the genes expressed exclusively in the mammary epithelium. Therefore the expression of the different genes has also been determined in two murine mammary epithelial cell lines, C57MG and RAC311, (see table 1) and will be analyzed in the pregnant mammary gland which contains much more epithelial tissue.

It was initially proposed to carry out this analysis by probing developmental Northern Blots of mammary gland mRNA. However we have since decided to use RT-PCR as it is more sensitive and specific. Primer pairs have been designed that specifically recognize *frizzled1-8*, *Notch1-4*, and *LRP5* and *LRP6* cDNAs using the published murine sequences, with the exception of *frizzled1* and *2*, and *frizzled5* for which the rat and human sequences were used respectively. The specificity of the primers has been confirmed by successful amplification of a band of the expected size from a cocktail of receptor cDNAs only when the sequence they were expected to amplify was present. Having established the specificity of the different pairs, we used them to analyze the expression of the different genes in the mature virgin mammary gland, and C57MG and RAC 311 cells by RT-PCR. The reactions were carried out using standard protocols and DNA contamination was controlled for by omitting reverse transcriptase from the RT reaction.

Gene	Virgin gland	RAC 311	C57MG
Fz1	-ve	-ve	-ve
Fz2	+ve (weak)	+ve	+ve
Fz3	-ve	-ve	-ve
Fz4	+ve	+ve	+ve
Fz5	+ve	+ve	+ve
Fz6	-ve	+ve	+ve
Fz7	-ve	-ve	-ve
Fz8	-ve	+ve	+ve
N1	-ve	+ve	+ve
N2	+ve	+ve	+ve
N3	-ve	-ve	-ve
N4	+ve	-ve	-ve
LRP5	+ve	+ve	+ve
LRP6	+ve	+ve	+ve

Table 1: Expression of the *frizzled*, *Notch* and *LRP* genes in the mature virgin mammary gland and murine mammary epithelial cell lines. The expression of the different genes was determined by RT-PCR. +ve indicates, that a particular gene is expressed in the tissue or cell line; –ve indicates that the expression was not detectable. These results are a summary of at least two consistent RT-PCR experiments.

II. Physical interaction between Wnt proteins and Frizzled, Notch and LRP proteins

In this section of the grant we proposed to examine the physical interaction between different Wnts and their potential receptors in cross-linking and co-immunoprecipitation experiments. The plasmids required for these experiments have been generated and the experiments are currently in progress.

We originally intended to carry out these experiments by co-expressing the Wnts with membrane tethered extracellular domains of the Frizzled, Notch or LRP proteins in 293T cells. However as all these proteins are cysteine-rich it is possible that the proteins will be inadvertently covalently linked to each other in the secretory pathway by inappropriate disulphide bonding when over expressed at high levels in the same cell. Such covalently linked

proteins would co-immunoprecipitate and erroneously suggest that they interact. To overcome this problem we decided to carry out these experiments by mixing two conditioned medium samples, one that contains the tagged Wnt protein and one that contains the Frizzled, Notch or LRP molecule. In this situation the two proteins are not expressed in the same cell removing the possibility that they will be inappropriately covalently linked to each other.

To generate conditioned media containing the Frizzled, Notch or LRP proteins, the protein must be secreted from the cell expressing it. Consequently tagged cDNAs that encode the extracellular domain only of the Frizzled, Notch or LRP protein have been constructed; the encoded proteins should be secreted as they contain a signal peptide but do not contain a transmembrane domain. These cDNAs have been cloned into pcDNA3 and the resultant plasmids have been transfected into 293T cells. In each case a protein of the expected size was expressed. However, with the exception of Frizzled8, LRP5 and LRP6, the proteins were not detectably secreted despite the lack of an obvious transmembrane domain and their detection in the endoplasmic reticulum by immunofluorescence (see figure 1 and data not shown). Therefore it will be difficult to complete these experiments using this approach.

Presently experiments are being conducted to identify the reason that these proteins are not secreted as we hope that this problem can be circumvented. In addition, cDNAs encoding truncated forms of the extracellular domains of Frizzled and Notch are being constructed in an effort to identify forms of the two proteins that are readily secreted from transfected cells. Finally an alternative strategy is being evaluated for the co-immunoprecipitation experiments. This involves co-culture of two cell lines expressing the Wnt proteins and either the Frizzled, Notch or LRP molecules. The cell mixture will then be lysed and immunoprecipitated. This approach avoids the problem of expressing the two proteins in the same cell and lysis of the cells will avoid problems with protein secretion. Others have used this approach successfully to show an interaction between the Frizzled1 protein and both Wnt3a and Wnt5a (Gazit et al., 1999).

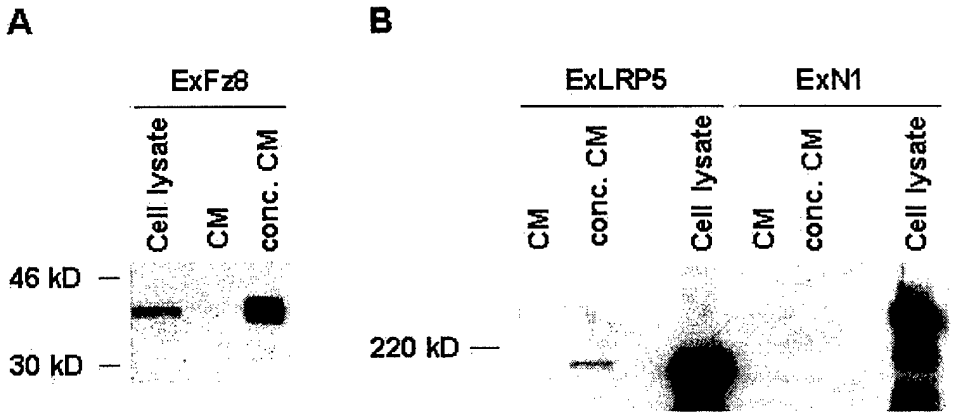

Figure 1: Secretion of the extracellular domains of the Frizzled, Notch and LRP proteins. 293T cells were transfected with pcDNA3 vectors containing cDNAs encoding the extracellular domains of the Frizzled8 (A), Notch1 (B) or LRP5 (B). Twenty four hours after transfection the cells were transferred into serum free medium and incubated for a further twenty four hours at 37°C. The medium was harvested from cells and concentrated twenty fold using a Centricom YM30 column. Samples of the concentrated and unconcentrated medium, along with SDS lysates of the transfected cells, were resolved by SDS PAGE and transferred to PVDF membrane. The proteins were then detected with the anti-myc antibody, 9E10. The extracellular domains of Frizzled8 (A) and LRP5 (B) are secreted into the conditioned medium. In contrast the Notch1 extracellular domain is not detectable in the medium although it is expressed in the cells (B).

III. Functional assays for the transduction of Wnt signal by Frizzled and Notch proteins

In the grant we proposed two functional assays, one using chimeric receptors and one using dominant negative receptors. The cDNAs encoding the rFrizzled1/Dfrizzled2 and mFrizzled8/Dfrizzled2 chimeric receptors have been synthesized and will be tested shortly. cDNAs encoding other chimeric receptors are currently being constructed. The dominant negative receptor experiments have yielded positive results and are described below.

Previous work in the laboratory has shown that the β-catenin protein accumulates in the cytoplasm of 293T cells when either they are treated with a conditioned medium containing a Wnt protein or transfected with a Wnt expression vector. However if a protein is expressed in these cells that disrupts Wnt signaling, β-catenin should no longer accumulate. We have used this assay to determine if the extracellular domains of the Frizzled, Notch and LRP proteins can disrupt Wnt signaling. Two slightly different approaches have been used in these experiments. In the first approach the 293T cells have been co-transfected with two plasmids, one that encodes a Wnt protein and one that encodes the Frizzled or Notch extracellular domain. Expression of the extracellular domain of the Frizzled5, Frizzled8 or Notch1-4 in 293T cells inhibits the

accumulation of β-catenin in response to Wnt1 (see figure 2 and data not shown). The Frizzled8 extracellular domain also inhibits the response of 293T cells to Wnt2, 3 and 3a.

In the second approach the 293T cells have treated with two conditioned medium samples, one containing the Wnt protein and one containing the extracellular domain of the Frizzled or LRP protein. This approach has been modified slightly to analyze whether the extracellular domain of Notch1 can inhibit Wnt signaling. As the extracellular domain of Notch1 is not secreted it is impossible to mix conditioned medium containing this protein with a Wnt containing medium. However the Notch protein is expressed on the surface of the cells allowing the Wnt medium to be pre-incubated with these cells before being added to the 293T cells. Consequently if the Notch1 molecule is able to sequester Wnt it will remove the Wnt protein from the medium before it is added to the 293T cells and reduce Wnt signaling. We found that the extracellular Frizzled8 and Notch1 domains can prevent Wnt signaling, Wnt1 and Wnt3a respectively, when mixed with a conditioned medium that contains a Wnt protein (see figure 2). Similar experiments are currently being carried out with conditioned medium containing the extracellular domain of LRP5.

In summary these results show that the extracellular domains of Frizzled8 and Notch1 can antagonize Wnt signaling in cell culture. These proteins may also inhibit Wnt signaling in vivo and therefore are potential candidates for expression in transgenic mice.

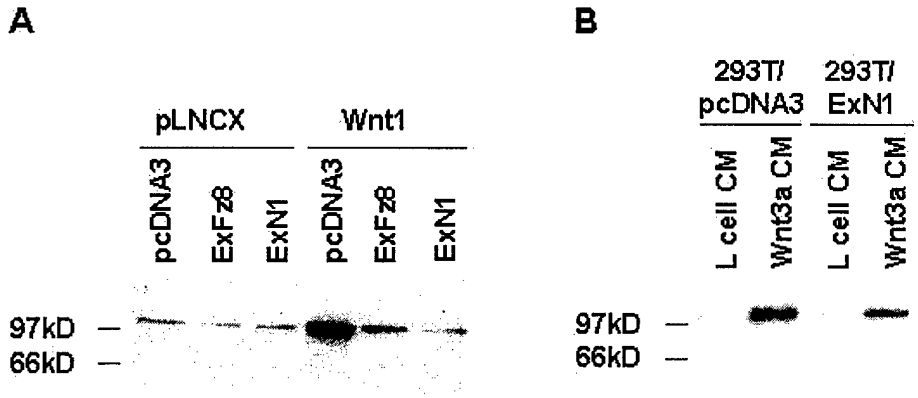

Figure 2: Inhibition of Wnt signaling by the extracellular domain of Frizzled8 and Notch1. (A) 293T cells were co-transfected with either a Wnt1 expression vector (lanes 4-6) or a control pLNCX vector (lanes 1-3), and either a pcDNA3 vector, which encodes the extracellular domain of Frizzled8 (lanes 2 and 5) or Notch1 (lanes 3 and 6), or a control pcDNA3 vector (lanes1 and 4) by calcium phosphate precipitation. 48 hours after transfection the cells were lysed. (B) 293T cells were treated with either Wnt3a conditioned medium (lanes 2 and 4) or control L cell conditioned medium (lanes 1 and 3) which had been pre-incubated for 30 minutes with either 293T cells expressing the extracellular domain of Notch1 (lanes 3 and 4) or control 293T cells (lanes 1 and 2). After 2 hours the cells were lysed.

The cell lysates were then fractionated into S100 and P100 fractions by high-speed centrifugation. Samples of the cytoplasmic fractions containing 5μg of total protein were resolved by SDS page and transferred to PVDF membrane. β-catenin was detected with an anti-β-catenin antibody. Expression of the extracellular domain of Frizzled8 or Notch1 in 293T cells prevents the accumulation of β-catenin in response to Wnt1 (compare lanes 4-6 in A). Similarly pre-incubation of Wnt3a conditioned medium with 293T cells expressing the Notch1 extracellular domain reduces Wnt signaling (compare lanes 2 and 4 in B). This suggests that the extracellular domains of Frizzled8 and Notch1 can act as dominant negative Wnt receptors.

IV. Generation of transgenic mice expressing dominant negative Wnt receptors

As the extracellular domain of the Frizzled8 protein is readily secreted and can inhibit Wnt1, 2, 3 and 3A signaling, we have decided to express this protein in transgenic mice. Expression will be targeted to the mammary gland by placing the cDNA encoding the Frizzled8 extracellular domain, with a myc tag at the C-terminus, under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). This has been done by cloning the cDNA into the pMSG (Pharmacia) vector that contains the MMTV LTR. The sequence of this construct has been verified and expression of the protein from the MMTV LTR has been confirmed in NIH3T3 cells (see figure 3). This transgene will now be introduced into Fvb mice by pronuclear injection.

The MMTV LTR contains a well defined glucocorticoid response element which can be used to drive the expression of a gene under the control of the enhancer in cells that express the glucocorticoid receptor such as NIH3T3 cells. Therefore, before introducing the transgene into mice we determined whether the Frizzled8 protein is expressed in response to dexamethasone. Two stably transfected NIH3T3 cell lines were generated, one that contains the Frizzled8 extracellular domain transgene and a control cell line that contains the original pMSG plasmid. Treatment of the cell line containing the transgene with dexamethasone, but not the ethanol solvent, induces expression of the Frizzled8 extracellular domain, while treatment of the control cell line does not lead to detectable expression of a detectable protein (see figure 3).

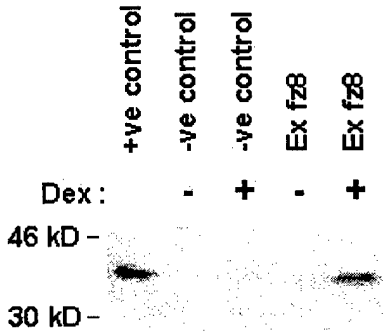

Figure 3: Expression of the extracellular domain of Frizzled8 under the control of the MMTV LTR. A stably transfected NIH3T3 cell line containing the Frizzled8 transgene (lanes 4 and 5) and a control cell line containing the original pMSG plasmid (lanes 2 and 3) were treated with 2μM dexamethasone dissolved in ethanol (lanes 3 and 5) or ethanol alone overnight (lanes 2 and 4). The cells were then lysed in Laemmli buffer. Samples of the lysates were resolved by SDS PAGE, transferred to PVDF membrane and the extracellular domain of the Frizzled8 was detected with the anti-myc antibody, 9E10. The Frizzled8 molecule was only expressed in the cell line containing the transgene when treated with dexamethasone (lane 5).

Key Research Accomplishments

- Identification of the Frizzled, Notch and LRP genes expressed in the mature virgin mammary gland and mammary epithelial cell lines.
- Generation of pcDNA3 plasmids containing tagged cDNAs of the extracellular domain of Frizzled, Notch or LRP family members for the co-immunoprecipitation experiments and functional assays.
- The extracellular domains of Frizzled8 and Notch1 have been shown to antagonize Wnt signaling in a functional assay for Wnt signaling making them potential candidates for expression in transgenic mice.
- Generation and testing of a mammary specific transgene for antagonizing Wnt signaling.

Reportable outcomes

None

Conclusions

A survey of the Frizzled, Notch and LRP proteins expressed in the mature virgin mammary gland of the mouse has been conducted to identify candidate Wnt receptors. The ability of these proteins to interact physically with Wnt ligands is currently being tested in cross-linking and co-immunoprecipitation experiments. Although problems have been encountered in

these experiments alternative approaches are currently being tried (see above). The ability of these proteins to transduce, and dominant negative forms to attenuate, a Wnt signal is being tested in a functional assay for Wnt signaling in cell culture. Finally a mammary specific transgene has been generated and is now ready for pronuclear injection to generate transgenic mice.

The purpose of the experiments in this grant is to identify receptor proteins responsible for the transduction of oncogenic Wnt signals in the mammary cells. Knowing the identity of mammary Wnt receptors is a crucial step in understanding the mechanisms by which the over-expression of Wnt proteins can result in tumorigenesis. In addition, the cell surface receptor proteins may be amenable targets for novel drug treatments and gene-based therapies aimed at preventing or treating breast cancer. Finally, work on many other signaling pathways has indicated that oncogenic mutations are frequently found in the genes encoding receptors for mitogenic growth factors. The analysis of Wnt receptors may therefore lead to identification of novel oncogenic mutations that contribute to breast cancer.

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Appedices

None